The Molecular Basis of Oligomeric Organization of the Human M₃ Muscarinic Acetylcholine Receptor

María José Varela Liste, Gianluigi Caltabiano, Richard J. Ward, Elisa Alvarez-Curto, Sara Marsango, and Graeme Milligan

Molecular Pharmacology Group, Institute of Molecular, Cell, and Systems Biology, College of Medical, Veterinary, and Life Sciences, University of Glasgow, Glasgow, Scotland, United Kingdom (M.J.V.L., G.C., R.J.W., E.A.-C., S.M., G.M.), and Laboratori de Medicina Computacional, Unitat de Bioestadística, Facultat de Medicina, Universitat Autònoma de Barcelona, Bellaterra, Spain (G.C.)

Received November 17, 2014; accepted March 13, 2015

ABSTRACT

G protein–coupled receptors, including the M₃ muscarinic acetylcholine receptor, can form homo-oligomers. However, the basis of these interactions and the overall organizational structure of such oligomers are poorly understood. Combinations of site-directed mutagenesis and homogenous time-resolved fluorescence resonance energy transfer studies that assessed interactions between receptor protomers at the surface of transfected cells indicated important contributions of regions of transmembrane domains I, IV, V, VI, and VII as well as intracellular helix VIII to the overall organization. Molecular modeling studies based on both these results and an X-ray structure of the inactive state of the M₃ receptor bound by the antagonist/inverse agonist tiotropium were then employed. The results could be accommodated fully by models in which a proportion of the cell surface M₃ receptor population is a tetramer with rhombic, but not linear, orientation. This is consistent with previous studies based on spectrally resolved, multiphoton fluorescence resonance energy transfer. Modeling studies furthermore suggest an important role for molecules of cholesterol at the dimer + dimer interface of the tetramer, which is consistent with the presence of cholesterol at key locations in many G protein–coupled receptor crystal structures. Mutants that displayed disrupted quaternary organization were often poorly expressed and showed immature N-glycosylation. Sustained treatment of cells expressing such mutants with the muscarinic receptor inverse agonist atropine increased cellular levels and restored both cell surface delivery and quaternary organization to many of the mutants. These observations suggest that organization as a tetramer may occur before plasma membrane delivery and may be a key step in cellular quality control assessment.

Introduction

G protein–coupled receptors (GPCRs) constitute the largest family of plasma membrane spanning polypeptides. They play pivotal roles in cell signaling and the regulation of biological processes and are privileged drug targets. The muscarinic receptors are a family (M₁–M₅) of GPCRs that respond to the neurotransmitter acetylcholine (Wess, 1996; Kruse et al., 2014). The M₃ receptor is involved in numerous important physiological functions. These include maintaining normal blood glucose levels by regulating insulin secretion (Ruiz de Azua et al., 2012; Nakajima et al., 2013) and the control of the salivary response (Ehlert et al., 2012; Sumida et al., 2013). Such functions reflect preferential activation of G proteins of the G₄ family (Wess, 1996; Kruse et al., 2014).

GPCRs were initially thought to exist and function exclusively as monomeric entities. However, evidence accumulated over the past two decades indicates that they may also form and function, at least in part, as dimers or higher order oligomers (Milligan, 2004, 2013; Ferre et al., 2014). The implications of such protein-protein interactions for regulating receptor trafficking and ligand pharmacology have been widely considered in recent times (Milligan, 2009; Lohse, 2010). Despite this, identification of the structural elements governing the dimerization/oligomerization of this class of receptors has been challenging (Milligan, 2013; Ferre et al., 2014). An understanding of the basis of such interactions is essential to better understand how GPCRs function at the molecular level. Such knowledge may provide new approaches to modulate GPCR oligomerization for therapeutic purposes. A series of studies has demonstrated the capacity of monomers of muscarinic receptor subtypes to self-associate in both living cells (Goin and Nathanson, 2006; Alvarez-Curto...
Materials and Methods

Materials for tissue culture were from Sigma-Aldrich (Poole, Dorset, UK), GE Healthcare (Little Chalfont, Buckinghamshire, UK), or Invitrogen (Paisley, UK). Rabbit polyclonal anti-SNAP tag antiserum was from New England BioLabs Inc. (Hitchin, UK). Anti-rabbit secondary IgG horseradish peroxidase–linked antibody was from GE Healthcare. Mouse monoclonal anti-α-tubulin antiserum was from Sigma-Aldrich. Anti-mouse secondary IgG horseradish peroxidase–linked antibody was from GE Healthcare. Hoechst 33342 trihydrochloride trihydrate was from Life Technologies (Paisley, UK). Oligonucleotides were from Thermo Fisher Scientific (Loughborough, UK) or Eurofins Genomics (Acton, London, UK). n-Dodecyl-β-D-maltoside (DDM) and atropine were from Sigma-Aldrich. NuPAGE Novex 4–12% Bis-Tris Gels, NuPAGE 4-morpholinepropanesulfonic acid SDS running buffer, and NativePAGE Novex 3–12% Bis-Tris gels were purchased from Life Technologies. Tag-lite reagents were supplied by Cibio Bioassays (Bagnols, Céze, France). Complete protease inhibitor cocktail tablets, N-glycosidase F, and DpnI restriction enzyme were from Roche Diagnostics (Burgess Hill, UK). [3H]-methylscopolamine ([3H]NMS) was from PerkinElmer (Buckinghamshire, UK).

DNA Constructs and Mutant Receptors. The plasmid pSEMI-26 m (SNAP tag), as supplied by Covalsy Biosciences AG/New England Biolabs (Hitchin, UK), was modified by the addition of a small linker region encoding the metabolotropic glutamate receptor 5 signal sequence (MVLLLILSSLLKEDYGRSAQS) and VSV-G epitope tag (YTDIEMNRGK) between the ClaI and EcoRI sites of the multiple cloning sites upstream of the SNAP tag (MCS1). hM3R was polymerase chain reaction (PCR) amplified using primers designed to add BamHI and NotI sites to the fragment termini and then ligated into the multiple cloning sites downstream of the SNAP tag (MCS2) of the modified plasmid (Alvarez-Curto et al., 2010b). This construct was used as a template to generate various mutants by alanine substitution mutagenesis using the QuikChange method (Stratagene, Agilent Technologies, Santa Clara, CA). Complementary primers containing the desired mutation, which was flanked by at least nine bases of wild-type sequence on either side, were used in the mutagenic PCR reactions. The PCR reaction mixture was digested with DpnI to remove the template DNA and leave only the newly synthesized double-stranded mutant construct, which was then transformed into a suitable bacterial host. The entire coding sequences of all mutant hM3 constructs were confirmed by sequencing.

Cell Culture and Transient Transfection of HEK293T Cells. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.292 g/L glucose, 100 U/ml penicillin, 0.1 mg/ml streptomycin mixture, and 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO2 humidified atmosphere. Cells were grown to 60–80% confluence in 60-mm dishes before transient transfection. For all experiments, transfections were performed with polyethylenimine (PEI) (Fluka Analytical, Poole, Dorset, UK). For homogeneous time-resolved (htr) FRET studies, a range of amount of DNA (0–2.5 μg) was combined with PEI (ratio 1:6) in 250 μL of 150 mM NaCl solution, thoroughly mixed, and incubated for 10 minutes at room temperature. The cell medium was then changed, and the DNA–PEI mixture was added to the cell medium in a dropwise manner.

Cell Treatments. For atropine treatment, transfected cells were incubated with 10 μM atropine for 24 hours.

[3H]N-Methylscopolamine Binding Assays. HEK293T cells transiently transfected with varying amounts (0–2.5 μg) of plasmid encoding VSV-SNAP-hM3R were grown overnight on white 96-well microtiter plates that had been treated with 0.1 mg/ml poly-l-lysine. The medium was removed and replaced with 100 μl per well cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM Na2HPO4, pH 7.4) containing 1 nM [3H]NMS. Nonspecific binding was determined in the presence of 10 μM atropine. The plates were incubated at 4°C for 150 minutes, and the assay was terminated by removal of the binding cocktail, followed by washing with 4 × 100 μl per well ice-cold 1× PBS. One hundred microliters per well of Microscint 20 (PerkinElmer) was added, and the plates were sealed before overnight incubation at room temperature on a rapidly shaking platform. Bound ligand was determined using a Packard TopCount NXT (PerkinElmer). Using the specific binding per well and number of cells per well, the receptor copies per cell were determined.

Cell Lysates and Western Blotting. Cells were washed once and harvested in cold PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM phosphate buffer saline (PBS)) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM Na2HPO4, pH 7.4) containing 1 nM [3H]NMS. Nonspecific binding was determined in the presence of 10 μM atropine. The plates were incubated at 4°C for 150 minutes, and the assay was terminated by removal of the binding cocktail, followed by washing with 4 × 100 μl per well ice-cold 1× PBS. One hundred microliters per well of Microscint 20 (PerkinElmer) was added, and the plates were sealed before overnight incubation at room temperature on a rapidly shaking platform. Bound ligand was determined using a Packard TopCount NXT (PerkinElmer). Using the specific binding per well and number of cells per well, the receptor copies per cell were determined.
Novex 3 additive (Life Technologies) was loaded into each lane of NativePAGE Lumi4Tb (10 nM), and Tag-lite SNAP-Red (100 nM) in a labeling mined optimal mixture of donor and acceptor, Tag-lite SNAP- incubated for 1 hour at 37°C in 5% CO2 in a humidified atmosphere SNAP-Lumi4Tb) in the labeling medium was added. Plates were probed with anti-SNAP antibody and developed as described above. Tween 20 at 4°C overnight on a rotating shaker. The membrane was then 15 minutes and blocked with 5% fat-free milk in PBS containing 0.1% seconds in methanol. The membrane was then fixed in 8% acetic acid for a polyvinylidene fluoride membrane that had been prewetted for 30 electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was then incubated in 5% fat-free milk in PBS containing 0.1% Tween 20 at 4°C overnight on a rotating shaker to block nonspecific binding sites. The membrane was incubated for 4 hours at room temperature with a polyclonal anti-SNAP tag antiserum diluted 1:2000, washed, and subsequently incubated with a horseradish peroxidase–linked anti-rabbit IgG secondary antibody (diluted 1:10,000) for 2 hours at room temperature.

Immunoblots were developed by the application of an enhanced chemiluminescence solution (Pierce Chemical, Rockford IL) according to the manufacturer's instructions. The membrane were stripped using standard protocol from Abcam (Cambridge, UK) and reprobed with anti-α-tubulin antiserum diluted 1:5000 for 4 hours at room temperature, washed, and subsequently incubated with a horseradish peroxidase–linked anti-mouse IgG secondary antibody (diluted 1: 20,000) for 2 hours at room temperature, before being developed as described above.

Treatments of Cell Lysates. Deglycosylation was performed using PNGlycosidase F at a final concentration of 0.05 U/μl for 2 hours at 37°C.

Blue Native PAGE. Cells transfected transiently with the appropriate plasmid were harvested in PBS and lysed in lysis buffer (150 mM NaCl, 0.01 mM Na2HPO4, pH 7.4, 2 mM EDTA, 0.5% DDM, and 5% glycerol) supplemented with complete protease inhibitor cocktail tablets (Roche Diagnostics) on a rotating wheel for 1 hour at 4°C. Samples were then centrifuged at 4°C for 15 minutes at 21,000g, and the supernatant was placed into fresh tubes. Laemmli loading buffer was added, and samples were heated at 65°C for 5 minutes and subjected to SDS-PAGE analysis using NuPAGE Novex 4–12% Bis-Tris gels and 4-morpholinepropanesulfonic acid SDS running buffer (Life Technologies). After electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was then incubated in 5% fat-free milk in PBS containing 0.1% Tween 20 at 4°C overnight on a rotating shaker to block nonspecific binding sites. The membrane was incubated for 4 hours at room temperature with a polyclonal anti-SNAP tag antiserum diluted 1:2000, washed, and subsequently incubated with a horseradish peroxidase–linked anti-rabbit IgG secondary antibody (diluted 1:10,000) for 2 hours at room temperature.

Results

Previous studies on MδR have shown that mutation of sequences within many of the TMD regions appears to compromise receptor quaternary structure formation or stability (McMillin et al., 2011). Such results have been interpreted as being consistent with the capacity of the receptor to form dimers in a range of ways via a number of distinct interfaces (McMillin et al., 2011; Hu et al., 2012). Recent studies have

Data Analysis. Experiments were performed on at least three independent occasions and analyzed using Prism 5.2 (GraphPad Software, La Jolla, CA). Where appropriate, data are expressed as mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance or Student’s t test.
suggested that rather than being limited to dimers, muscarinic receptors, including both the M₂ (Redka et al., 2014) and M₃ (Patovery et al., 2013) subtypes, may form and potentially function as tetramers. We therefore initiated studies to address such a possibility for structural organization and assess if previous results (McMillin et al., 2011) would be consistent with such a model.

To explore the contribution of various regions of the sequence of hM₃R to self-association and organization into dimers and/or higher oligomers, we modified this receptor to optimize cell surface delivery in transient transfection studies. These alterations were also designed to produce a version of the receptor that is suitable for both immunodetection and allow covalent attachment of fluorophores to appropriately folded and cell surface–delivered copies of the protein. The first of these requirements was achieved by the addition of the signal sequence from metabotropic glutamate receptor 5 to the extracellular N-terminal domain of the receptor, as we have previously used for a number of other rhodopsin-like GPCRs (Xu et al., 2012). Additional incorporation of the VSV epitope tag sequence and then the SNAP-tag polypeptide after the signal sequence provided the other two desired features and produced the final wild-type construct VSV-SNAP-hM₃R. This reflects that the N-terminal domain of GPCRs will be extracellular if the protein is correctly delivered to the plasma membrane. Using intact cells, only cell surface copies of the receptor can be therefore labeled by SNAP tag–targeted fluorophores (Ward and Milligan, 2014). cDNA encoding VSV-SNAP-hM₃R was transfected transiently into HEK293T cells. Subsequent immunoblotting of SDS-PAGE resolved cell lysates with an anti-SNAP antiserum, which resulted in detection of the translated products as a group of poorly resolved species. These alterations were also designed to produce a version of the receptor that is suitable for both immunodetection and allow covalent attachment of fluorophores to appropriately folded and cell surface–delivered copies of the protein. The first of these requirements was achieved by the addition of the signal sequence from metabotropic glutamate receptor 5 to the extracellular N-terminal domain of the receptor, as we have previously used for a number of other rhodopsin-like GPCRs (Xu et al., 2012). Additional incorporation of the VSV epitope tag sequence and then the SNAP-tag polypeptide after the signal sequence provided the other two desired features and produced the final wild-type construct VSV-SNAP-hM₃R. This reflects that the N-terminal domain of GPCRs will be extracellular if the protein is correctly delivered to the plasma membrane. Using intact cells, only cell surface copies of the receptor can be therefore labeled by SNAP tag–targeted fluorophores (Ward and Milligan, 2014). cDNA encoding VSV-SNAP-hM₃R was transfected transiently into HEK293T cells. Subsequent immunoblotting of SDS-PAGE resolved cell lysates with an anti-SNAP antiserum, which resulted in detection of the translated products as a group of poorly resolved species (Fig. 1A). Treatment with N-glycosidase F showed these to reflect the differential extents of N-glycosylation of the receptor polypeptide (Fig. 1A), in which the predominant forms migrated with an apparent molecular mass of between 100 and 120 kDa. Effective cell surface delivery of the VSV-SNAP-hM₃R construct was demonstrated following addition to intact cells of SNAP-Lumi4Tb by monitoring fluorescence emission at 620 nm after excitation at 337 nm (Fig. 1B). This fluorophore binds covalently to the SNAP tag within the extracellular N-terminal domain of the receptor construct in a 1:1 ratio. Increasing levels of 620-nm emission and, therefore, cell surface receptor per cell, were recorded as the amount of transfected cDNA was increased (Fig. 1B). Levels of cell surface VSV-SNAP-hM₃R expression were quantified by the specific binding of the cell impermeant, muscarinic antagonist [³H]NMS. This also increased with the amount of cDNA transfected and corresponded to some 20,000–200,000 copies per cell over the range of cDNA amounts used (Fig. 1C). Demonstration that the receptor construct was forming dimeric/oligomeric complexes at the surface of the cells was produced by coaddition of a single concentration of SNAP-Lumi4Tb as an energy donor to intact cells with varying concentrations of SNAP-Red as an energy acceptor. This resulted in a bell-shaped distribution of resonance energy transfer from SNAP-Lumi4Tb to SNAP-Red and subsequent emission at 665 nm, following excitation at 337 nm, in htrFRET experiments (Fig. 1D). This outcome was anticipated because competition between the energy donor and acceptor to covalently label the available SNAP tag–containing cell surface hM₃R population eventually results in the energy acceptor outcompeting the energy donor. This limits resonance energy transfer at high acceptor concentrations (Ward and Milligan, 2014). Importantly, such preliminary experiments also identified the optimal ratio of SNAP-Lumi4Tb to SNAP-Red to maximize receptor-receptor htrFRET signals for subsequent studies (Fig. 1D). To define that the htrFRET output at 665 nm actually reported hM₃R-hM₃R interactions and not simply protein–protein proximity because of the amount of receptor expressed, we employed the single TMD epidermal growth factor receptor. In the absence of agonist activation, this receptor is almost entirely monomeric. The extracellular N-terminal region of this receptor was also modified to incorporate both the VSV and SNAP tags. Transient transfection was optimized to achieve a similar cell surface expression level of this construct as for VSV-SNAP-hM₃R, which was measured by emission at 620 nm following addition of SNAP-Lumi4Tb (Fig. 1D, insert). However, now coaddition of a range of concentrations of SNAP-Red resulted in very little energy transfer (Fig. 1D).

Biochemical analysis also indicated that VSV-SNAP-hM₃R was present within oligomeric complexes. The addition of DDM extracts of lysates of VSV-SNAP-hM₃R expressing cells to nondenaturing Blue Native PAGE resulted in the resolution and detection of a high molecular mass and apparently higher order complex species of VSV-SNAP-hM₃R (Fig. 1E). As also shown previously for a related hM₃R construct (Milligan, 2013), these oligomeric forms were separated into differentially N-glycosylated forms of monomeric VSV-SNAP-hM₃R when SDS (1% (w/v)) was added to the samples prior to addition to the Blue Native gel (Fig. 1E). This indicates that the higher order complexes were not covalently linked adducts and did not represent irreversibly aggregated protein. Importantly, over the full range of cDNA amounts employed, the level of cell surface–receptor oligomers, detected as an htrFRET output at 665 nm after coaddition of the optimal ratio of SNAP-Lumi4Tb and SNAP-Red, increased in a linear manner with the amount of cell surface–delivered receptor, as measured by fluorescence emission at 620 nm (Fig. 1F).

To identify TMD and/or helix VIII residues involved in hM₃R homomeric interactions, the VSV-SNAP-hM₃R construct was used as a template to generate a series of variants by alanine mutagenesis (Fig. 2). These ranged from forms that mutated a pair of amino acids to more extensive alterations involving concurrent replacement of up to four distinct residues. Residues mutated within these regions were selected based, in part, on inspection of the atomic level structure of the rat M₃ receptor bound by the high affinity antagonist/inverse agonist tiotropium (Kruse et al., 2012). Initially, we assessed cell surface delivery of the mutant receptors by emission at 620 nm after excitation at 337 nm in transiently transfected HEK293T cells that were incubated with SNAP-Lumi4Tb (Fig. 3A). The majority of the mutants displayed reduced, and in some cases very poor, delivery to the cell surface compared with the wild-type VSV-SNAP-hM₃R sequence (Fig. 3A).

Furthermore, as also noted by others (McMillin et al., 2011), the mutants that displayed the poorest cell surface delivery also showed poor levels of the mature, more fully N-glycosylated and, therefore, lower mobility species. In such cases, the bulk of the immunodetected protein of these mutants was a poorly N-glycosylated, approximately 85-kDa form (Fig. 3, B and C). Indeed, in examples, such as the TMD VI mutant I502A, I509A, V513A, F516A VSV-SNAP-hM₃R and TMD VII mutant F525A, L528A, L532A, I535A VSV-SNAP-hM₃R,
Fig. 1. At the cell surface, VSV-SNAP-hM₃R is an N-glycosylated, oligomeric complex. (A) Lysates from HEK293T cells transfected with empty vector or VSV-SNAP-hM₃R were resolved by SDS-PAGE with or without pretreatment with N-glycosidase F (PNGaseF) and immunoblotted with either anti-SNAP antiserum (upper panel) or anti-α-tubulin as a loading control (lower panel). (B) Cells transfected with empty vector or varying amounts of VSV-SNAP-hM₃R containing plasmid for 24 hours were incubated with the htrFRET energy donor (SNAP-Lumi4Tb, 10 nM). Cell surface binding of this ligand was determined by fluorescent emission at 620 nm after excitation at 337 nm and standardized for cell number. (C) Cells as in (B) were used to measure the specific binding of a single concentration of [³H]NMS, which was estimated to occupy greater than 95% of the receptor. (D) Cell surface oligomeric interactions of VSV-SNAP-hM₃R were defined by htrFRET. Optimal concentrations of the SNAP-tag htrFRET energy donor and acceptor were established by incubating cells with a fixed concentration of energy donor (SNAP-Lumi4Tb, 10 nM) and various energy acceptor (SNAP-Red) concentrations. htrFRET measured at 665 nm after excitation at 337 nm reflects the proximity between labeled copies of VSV-SNAP-hM₃R at the cell surface (circles). Equivalent experiments were performed on cells expressing the single TMD VSV-SNAP–epidermal growth factor receptor (squares) at equal levels of cell surface expression, as defined by binding and emission at 620 nm of SNAP-Lumi4Tb (insert; open bars = mock transfection; filled bars = corresponding receptor). (E) DDM extracts of lysates from HEK293T cells transfected with empty vector or VSV-SNAP-hM₃R were prepared and treated or not with 1% SDS before resolution by Blue Native PAGE and immunoblotted with the anti-SNAP antiserum. (F) HEK293T cells were transfected with increasing amounts of VSV-SNAP-hM₃R for 24 hours and then incubated with the optimal combination and ratio of SNAP-Lumi4Tb (10
where cell surface delivery was almost extinguished, this 85-kDa species was the only form of the receptor construct detected by the anti-SNAP antiserum (Fig. 3C).

Although many of the mutants were expressed modestly at the cell surface compared with the wild-type receptor when using the same amount of cDNA, for each mutant, the level of cell surface delivery per cell also increased in a linear manner with an increasing amount of cDNA used for transfection. Therefore, by employing a range of amounts of cDNA, direct comparisons of the effectiveness of interactions of the mutated receptors could be made with wild type at equal cell surface expression levels. This was achieved by measuring the slope of the linear regression of the line for the htrFRET signal at 665 nm versus the expression level, which was measured as the signal at 620 nm following addition of only SNAP-Lumi4Tb, and comparing this for each individual mutant to data produced for wild-type VSV-SNAP-hM3R in parallel studies (see Fig. 4 for the entire data set).

Both in previous mutagenesis studies of hM3R (McMillin et al., 2011) and X-ray structure dimer interfaces for the β1-adrenoceptor (Huang et al., 2013) and µ-opioid receptor (Manglik et al., 2012), residues near the cytoplasmic face of TMD V are indicated to play important roles in receptor-receptor interactions. Herein, concerted mutation to alanine of W252, R253, and Y255 [residue positions 5.59, 5.60, and 5.62 in the Ballesteros and Weinstein (1995) positioning system] located at the intracellular end of TMD V (Fig. 5A) generated a form of the receptor that was poorly expressed at the cell surface compared with wild type. However, this mutant was still able to produce homomeric interactions, as assessed by htrFRET emission at 665 nm after coaddition of SNAP-Lumi4Tb and SNAP-Red (Fig. 5B). These interactions were significantly (Fig. 4) reduced (Fig. 5B), however, compared with wild type. Limiting the TMD V mutations to only W252A and R253A resulted in a mutant that was better expressed at the cell surface than W252A, R253A, Y255A VSV-SNAP-hM3R (Figs. 3, A and B, and 5B), but which was equally as compromised in oligomeric interactions as W252A, R253A, Y255A VSV-SNAP-hM3R (Figs. 4 and 5B). These results demonstrate, as indicated previously (McMillin et al., 2011), an important role of this region in hM3R homomeric interactions.
Equivalent studies were performed for all the other mutants detailed in Fig. 2. Combined mutation of I502A and I509A (residues 6.46 and 6.53) or V513A and F516A (residues 6.57 and 6.60) in TMD VI produced forms of VSV-SNAP-hM3R that each had major defects in oligomeric interactions (Figs. 4 and 6A). Moreover, both were markedly compromised in cell surface delivery (Fig. 3). Combining these mutations resulted in a version of the receptor that was immature, lacked significant N-glycosylation, and was very poorly expressed at the cell surface (Fig. 3). However, to the extent that htrFRET signals could be measured at the cell surface for this mutant (Fig. 6A), it appeared to be no more greatly compromised in receptor-receptor interactions than either of the two double mutants that were combined to generate this form (Fig. 6A; see Fig. 4 for quantification). Mutations introduced in TMD VII between amino acids 525 (residue 7.34) and 535 (residue 7.44), upstream of the NPXXY sequence (NPVCY in hM3R) that is highly conserved in rhodopsin-family GPCRs, were next assessed. F525A, L528A VSV-SNAP-hM3R behaved in a manner indistinguishable from wild type (Figs. 4 and 6B). By contrast, L532A, I535A VSV-SNAP-hM3R was markedly compromised in its capacity for oligomeric assembly (Figs. 4 and 6B) and was poorly expressed and delivered to the cell surface. Unsurprisingly, based on these observations, the combined F525A, L528A, L532A, I535A VSV-SNAP-hM3R was also very poor in expression and oligomeric assembly (Fig. 6B; see Fig. 4 for quantitative details).

A number of regions within TMD I have been suggested as contributing to potential dimer/oligomer interfaces from each of the experimental, structural, and computational approaches.
Herein, residues within the sequence from Q67 (residue location 1.31) at the extreme extracellular end to I87 (residue location 1.51) were modified in various combinations. All residues on the outward face of these areas were mutated to assess their potential involvement, as observed in crystals of the m- (Manglik et al., 2012) and k-opioid (Wu et al., 2012) receptors and turkey β1-adrenoceptor (Huang et al., 2013). Beginning at the extracellular end of TMD I, mutation of residues to produce Q67A, V68A, I71A, L74A VSV-SNAP-hM3R caused a relatively small, although highly statistically significant (Fig. 4), effect on receptor organization (Fig. 7A). Combined alanine mutation of residues V69, F73, I77, and L80, each also lying on the outward-facing side of this helix, had no significant effect (Fig. 4) on oligomeric organization (Fig. 7B). However, this combination of mutations had a marked effect on construct expression (Fig. 3). Deconstruction of this variant to firstly generate V69A, F73A VSV-SNAP-hM3R allowed effective expression and cell surface delivery (Fig. 3) of a receptor that also behaved as wild type in the oligomerization assay (Fig. 4; Fig. 7B). I77A, L80A VSV-SNAP-hM3R was expressed as poorly at the cell surface as the quadruple mutant (Fig. 3), but again, although producing only a modest effect on receptor oligomerization (Fig. 7B), this was statistically significant (Fig. 4). By contrast, mutation of residues further down TMD I to produce L80A, I83A, I84A, I87A VSV-SNAP-hM3R had a large effect on both cell surface expression

![Fig. 4. Many regions of the helical domains of hM3R contribute to effective oligomerization. htrFRET experiments for each mutant studied, including those shown in Figs. 5–9, were analyzed by linear regression. The slope values of 665 nm over 620 nm fluorescence emission, as defined in Figs. 5–9, were then normalized relative to the slope value obtained with VSV-SNAP-hM3R (which was included as control in each individual experiment). Data are mean ± S.E.M. of at least three independent experiments. ***P < 0.001 and *P < 0.05, one-way analysis of variance compared with VSV-SNAP-hM3R.](image1)

**Fig. 4.** Many regions of the helical domains of hM3R contribute to effective oligomerization. htrFRET experiments for each mutant studied, including those shown in Figs. 5–9, were analyzed by linear regression. The slope values of 665 nm over 620 nm fluorescence emission, as defined in Figs. 5–9, were then normalized relative to the slope value obtained with VSV-SNAP-hM3R (which was included as control in each individual experiment). Data are mean ± S.E.M. of at least three independent experiments. ***P < 0.001 and *P < 0.05, one-way analysis of variance compared with VSV-SNAP-hM3R.

![Fig. 5. Mutation of residues at the intracellular end of TMD V of hM3R alters both expression and oligomeric interactions. (A) Representation of the primary sequence of TMD V from hM3R indicated by the one-letter amino acid code, with residue numbering of amino acids that were replaced with alanine shown with a dark background and position in the primary sequence of hM3R. Proline residue 5.50 is also highlighted. The residues mutated are shown as sticks in the cartoon representation of hM3R. (B) Representative htrFRET assays performed in HEK293T cells transfected with differing amounts of VSV-SNAP-hM3R (circles), W252A, R253A VSV-SNAP-hM3R (squares), or W252A, R253A, Y255A VSV-SNAP-hM3R (triangles). Following incubation with a combination of SNAP-Lumi4Tb (10 nM) and SNAP-Red (100 nM), both fluorescence emission at 620 nm and the htrFRET signal (fluorescence emission at 665 nm) were measured. The plot shown was analyzed by linear regression.](image2)

**Fig. 5.** Mutation of residues at the intracellular end of TMD V of hM3R alters both expression and oligomeric interactions. (A) Representation of the primary sequence of TMD V from hM3R indicated by the one-letter amino acid code, with residue numbering of amino acids that were replaced with alanine shown with a dark background and position in the primary sequence of hM3R. Proline residue 5.50 is also highlighted. The residues mutated are shown as sticks in the cartoon representation of hM3R. (B) Representative htrFRET assays performed in HEK293T cells transfected with differing amounts of VSV-SNAP-hM3R (circles), W252A, R253A VSV-SNAP-hM3R (squares), or W252A, R253A, Y255A VSV-SNAP-hM3R (triangles). Following incubation with a combination of SNAP-Lumi4Tb (10 nM) and SNAP-Red (100 nM), both fluorescence emission at 620 nm and the htrFRET signal (fluorescence emission at 665 nm) were measured. The plot shown was analyzed by linear regression.
Indeed, in the case of M₃R, residues of TMD IV located toward the cytoplasmic face have been previously shown to limit dimeric/oligomeric organization (McMillin et al., 2011). We also generated mutants, therefore, in both this region (K183A, R184A, V187A VSV-SNAP-hM₃R) and higher up TMD IV (L191A, V194A, I195A, V198A VSV-SNAP-hM₃R). Although L191A, V194A, I195A, V198A VSV-SNAP-hM₃R did not alter oligomeric organization (Figs. 4 and 8A), as predicted from previous studies, K183A, R184A, V187A VSV-SNAP-hM₃R displayed markedly reduced interactions (Figs. 4 and 8B).

Residues within VIII are often noted to contribute to the same receptor-receptor interaction interface as amino acids in TMD I (Milligan, 2013; Ferre et al., 2014). Therefore, residues within the helix VIII section of the C-terminal tail, which runs parallel to the plasma membrane, were altered. Mutation of both L559 (residue location 8.58) and L560 (residue location 8.59) just before the end of this helix (Fig. 9A) resulted in both reduced expression (Fig. 3) and markedly reduced oligomer interactions (Figs. 4 and 9B). Moreover, mutation of a series of residues located in a region more proximal to TMD VII to produce F555A, K556A, M557A VSV-SNAP-hM₃R also resulted in a form of the receptor in which more modest, but still significant (Fig. 4), reduction in the effectiveness of cell surface receptor-receptor interactions was recorded (Fig. 9C).

A series of studies have indicated that receptor dimerization/oligomerization may occur before membrane delivery, potentially in the endoplasmic reticulum (ER)/Golgi apparatus as part of the quality control of protein folding and maturation (Salahpour et al., 2004; Wilson et al., 2005; Canals et al., 2009; Kobayashi et al., 2009). Many GPCR mutants are incorrectly organized and sent for destruction prior to cell surface delivery. In a number of cases, including receptor variants that appear poorly able to dimerize/oligomerize (Dunham and Hall, 2009; Leidenheimer and Ryder, 2014), pharmacological ligands that can cross cellular membranes are able to bind to the mutant receptor at the ER/Golgi and promote (more) effective folding and subsequent cell surface delivery (Dunham and Hall, 2009; Leidenheimer and Ryder, 2014). Ligands acting in this manner are described as pharmacological chaperones (Dunham and Hall, 2009; Leidenheimer and Ryder, 2014; Petaja-Repo and Lackman, 2014). To assess ligand effects on a number of the mutant forms of VSV-SNAP-hM₃R, we employed sustained overnight treatment of HEK293T cells transiently expressing the variant of interest with the prototypic high affinity muscarinic antagonist/inverse agonist atropine. Even for the wild-type VSV-SNAP-hM₃R construct, atropine treatment resulted in somewhat higher levels of the cell surface receptor, as monitored by the binding of SNAP-Lumi4Tb (Fig. 10A). Moreover, across a range of amounts of cDNA transfected, the slope of the 665-nm htrFRET emission signal against 620-nm cell surface expression levels was increased (Fig. 10B), suggesting a closer distance between the energy donor and acceptor forms when the receptor was occupied with this ligand. As shown earlier, the TMD I mutant L80A, I83A, I84A, I87A VSV-SNAP-hM₃R was poorly expressed (Fig. 3), but after sustained treatment with atropine, cell surface expression was improved significantly (Fig. 10B). Notably, moreover, the slope of the 665-nm htrFRET emission signal against 620-nm cell surface expression levels increased markedly for this mutant to become indistinguishable from wild type (Fig. 10B). This suggests that following atropine binding, the organizational structure of the
receptor oligomer of this variant also adopted a state akin to the wild-type receptor. Equally, for the TMD V mutant W252A, R253A VSV-SNAP-hM3R, atropine treatment improved cell surface delivery (Fig. 10A) and receptor organizational structure (Fig. 11A). Analysis across a broad range of the mutants studied indicated that for the majority, treatment with atropine increased cell surface delivery and improved receptor surface organization to a state akin to wild type (Fig. 11A). However, this was not universal. For the TMD VI mutants, although V513A, F516A VSV-SNAP-hM3R displayed this behavior, the more extensive I502A, I509A, V513A, F516A VSV-SNAP-hM3R mutant did not show this pattern, with no increase in the 665/620 nm slope following treatment with atropine (Fig. 11A). To explore this further, immunoblots of untreated and atropine-treated cells expressing the TMD VI mutants were compared. Interestingly, although the I502A, I509A, V513A, F516A VSV-SNAP-hM3R mutant displayed no mature protein in the absence of atropine treatment, ligand treatment did result in a proportion of the receptor now becoming N-glycosylated, and the proportion of mature I502A, I509A VSV-SNAP-hM3R and V513A, F516A VSV-SNAP-hM3R was increased (Fig. 11B). This was also the case for the TMD VII mutant F525A, L528A, L532A, I535A VSV-SNAP-hM3R (not shown).

The overall data set indicated regions in TMD I, TMD IV, TMD V, TMD VI, and TMD VII as well as helix VIII, which affected receptor homomeric interactions. To consider ways to integrate these results with those of others (Park et al., 2002; McMillin et al., 2011; Patowary et al., 2013), we employed an inactive state X-ray structure of the rat M3 muscarinic receptor monomer bound by the antagonist tiotropium (Kruse et al., 2012). This was used to generate models of a potential dimeric/oligomeric organizational structure that would predict further interfaces and would be consistent with the experimental data set (Fig. 12). Fitting together crystal structure monomers of M3R, based on the dimensions of the protein and complementarity of shape, allowed a tightly packed rhombic tetramer model to be produced (Fig. 12A). This generated a complex with potential contributions from selected residues of TMDs V–VII at the dimer + dimer interface (Fig. 12A). Other oligomer models based on a TMD I, TMD II, and helix VIII dimer interface could also be generated. These included a linear model (Fig. 12B) that resulted in further interfaces that were somewhat akin to those either observed directly or which can be postulated from GPCR crystal structures. It was also possible to generate a square tetramer model (Fig. 12C), but this resulted in a large gap at the center of the complex that seemed energetically unlikely. A further feature of the higher order models based on the TMD I, TMD II, and helix VIII dimer interface was that this rhombic model also allowed simultaneous interactions with two heterotrimeric G proteins in their vicinity.
nucleotide-free form (Supplemental Fig. 1). By contrast, when we attempted to generate rhombic models that required a pivotal role of TMD V at the interface of each dimeric unit, it was not possible to either dock two heterotrimeric G proteins in a manner that avoided steric clashes or could account for the experimental data that allowed discrimination between the models (see Supplemental Figs. 1–3 and Discussion).

Discussion

The capacity of class A GPCRs to generate dimers and/or higher order oligomers is well appreciated (Milligan, 2013; Ferre et al., 2014), as is the potential importance of this for function (Milligan, 2004, 2013; Ferre et al., 2014). However, the basis of how monomers form dimers and, indeed, how dimers might interact to generate higher order complexes and the interfaces involved are a matter of considerable debate (Milligan, 2013; Ferre et al., 2014). Data from Wess et al. have shown that hM₃R can generate sufficiently extensive homomeric interactions to allow capture of dimeric forms via chemical cross-linking (Hu et al., 2012). Mutation across individual TMD regions of this receptor has shown that a number of regions contribute to dimeric or higher order organization (McMillin et al., 2011). Clearly, there are a number of ways in which such results might be interpreted. One is that dimeric interactions may be able to occur in a number of distinct ways, including via each of the TMD V–TMD V, TMD VI–TMD VII, TMD IV–TMD V, and TMD I–TMD II interactions (McMillin et al., 2011).

Observations that dimer/oligomer interactions of muscarinic receptor subtypes may be transient (Hern et al., 2010; Nenasheva et al., 2013) are certainly consistent with this concept if it predominantly reflects a “kiss and run” phenomenon (Milligan, 2000). A second interpretation of the results of both Wess et al. (McMillin et al., 2011) and those we report herein is that the contributions of multiple regions of the receptor to structural organization reflects that higher order complexes beyond dimers can form. This would require contributions from multiple regions of the receptor and might increase complex stability. It is noteworthy, therefore, that although mutations in a number of regions of the receptor did alter htrFRET signals in a manner consistent with producing changes in the overall organizational structure, no single mutant or set of mutants appeared to prevent receptor-receptor contacts. This was also the case in the studies of McMillin et al. (2011). Within the current studies and as shown previously for other hM₃R constructs (Milligan, 2013), resolution of cell extracts on non-denaturing gels showed a high proportion of the receptor to be present in larger complexes. These were converted to monomeric forms by preaddition of SDS. In support of this reflecting noncovalent oligomerization, imaging studies linked to mathematical analysis have suggested that a substantial proportion of hM₃R can exist as tetramers at the cell surface (Patowary et al., 2013). Moreover, these studies suggested a potential dynamic interchange between these tetramers and dimer states (Milligan, 2013; Patowary et al., 2013). This may be relevant to the observations both herein and in the studies of McMillin et al. (2011) of a clear role for amino acids at the cytoplasmic end of TMD IV, as this is not inherently predicted by the rhombic tetramer models. Organization of other class A GPCRs as tetramers has also been supported by approaches, including reconstitution experiments in artificial lipid bilayers (β₂-adrenoceptor) (Fung et al., 2009), chemical cross-linking studies (dopamine D₄) (Guo et al., 2005), and detailed characterization of ligand binding studies (muscarinic M₂) (Park et al., 2002; Redka et al., 2014).

Both rhombic and linear tetramer models of hM₃R that employed an interface involving residues from TMD I and intracellular helix VIII could be produced. Each of these resulted in a second interface that predicted contributions from residues from TMD V and TMD VI. The most extensive predicted dimer + dimer interface in the rhombic tetramer model involved TMD VI, TMD VII, and part of TMD I (Fig. 12). No role of TMD VII in the quaternary structure was implicated from the linear tetramer model, and the same was the case for the outer residues of TMD VI close to TMD VII. This allowed experimental assessment of the linear versus...
rhombic tetramer models, with markedly different predicted outcomes for mutants in TMD VI and TMD VII on htrFRET signals and quaternary structure. Mutations in TMD VII upstream from the NPXXY domain resulted in large effects on htrFRET-detected oligomer organization. Such observations are, at a minimum, consistent with the rhombic tetramer model, but not with the linear one. However, despite these experimental results, the X-ray structure of the M₃ receptor clearly shows that TMD VII is a strongly kinked helix and shaped concavely (Kruse et al., 2012). As such, it appears ill suited to make extended direct protein-protein and helix-helix interactions in either a dimer or a higher order complex. Furthermore, none of the released crystal structures of GPCR dimers involves TMD VII. However, although the complementarity of the shape of M₃R monomers and dimers allowed construction of conceptual rhombic tetramer models, it should be noted that, although much less obvious and extensive than in the square tetramer model, this organization also resulted in a space between the dimers in the region of the proposed dimer + dimer interface, which was close to residues from TMD I, TMD VI, TMD VII, and helix VIII (Fig. 12). Such protein-only initial models of receptor organization did not consider the possible contributions of lipids. Many released crystal structures of class A GPCRs, including the β₂-adrenoceptor (Cherezov et al., 2007), serotonin 5-HT₂B receptor (Wacker et al., 2013), adenosine A₂A receptor (Jaakola et al., 2008), μ-opioid receptor (Manglik et al., 2012), and P₂Y₁₂ receptor (Zhang et al., 2014), contain molecules of cholesterol. Moreover, cholesterols are sometimes present at the same positions in different crystals. For example, cholesterols at TMD I are observed in both the β₂-adrenoceptor (Cherezov et al., 2007) and serotonin 5-HT₂B receptor (Wacker et al., 2013) crystals. Equally, a cholesterol molecule is positioned at the extracellular side of TMD VI–TMD VII in both the adenosine A₂A receptor (Jaakola et al., 2008) and μ-opioid receptor (Manglik et al., 2012) structures. Moreover, the same cholesterol, after building the rhombic tetramer hM₃R models, superimposed well with the cholesterol observed in the extracellular side of TMD VII of the P₂Y₁₂ receptor (Zhang et al., 2014). In addition to such direct observations, Cang et al. (2013) have computationally predicted cholesterol to be in a similar position in the β₂-adrenoceptor. We therefore specifically introduced these cholesterols into the models (Fig. 13). In such detailed models of the rhobic tetramer (Fig. 13), molecules of cholesterol bridge the dimer + dimer interface to fill the gap. The model that was built to describe the experimental mutagenesis results has two cholesterols interacting with TMD VI near the extracellular side, which, in the rhobic tetramer constructs, create a layer of four cholesterol molecules that line up to form a buffer between the dimers (Fig. 13). These mediate interactions of TMD VII with TMD VI as well as with residues from TMD I. This model is consistent with data from the TMD VII mutant F525A, L528A, L532A, I535A hM₃R. This mutant had a dramatic effect on the quaternary structure. Even limiting the alterations to a pair of amino acids (L532A, I535A) (positions 7.41 and 7.44) had an equally dramatic effect. It is noteworthy that McMillin et al. (2011) also reported that a mutation of hM₃R encompassing residues L532A and I535A resulted in substantial disruption of the quaternary structure. However, although McMillin et al. (2011) suggested a TMD VII–TMD VI protein-protein contact interface as one possible dimeric arrangement based on these
results, we offer a very different interpretation that is also consistent with the X-ray structure of \( \text{M}_{2}\text{R} \) (Kruse et al., 2012). This structure shows that L532A and I535A lie deep in the concave spot of helix TMD VII, a location from which they would be unlikely to form direct residue-residue interactions with TMD VI. However, our model predicts they can do so via the tail of an intermediate molecule of cholesterol (Fig. 13). This level of structural detail was not available at the time of the report of McMillin et al. (2011). By contrast, limiting the TMD VII mutation to only F525A and L528A generated a receptor variant that was well expressed and displayed no significant deficit in quaternary organization. The rhombic tetramer model also predicts an important role for TMD V. However, rather than at the monomer-monomer interface of the individual dimers, herein, the role of TMD V residues is at the dimer + dimer interface. Interestingly, therefore, a clear effect on receptor organization was produced in the TMD V W252A, R253A hM3R receptor mutant. The model shows cholesterol binding to the lower part of TMD I, mediating an interaction of TMD I from one dimer with TMD V from the second dimer and specifically involving residues W252 and R253 (Fig. 13).

Although inherently speculative, the concept that molecules of cholesterol may play integral roles in receptor organization is encouraged from the atomic level structure of the \( \beta_2\)-adrenoceptor (Cherezov et al., 2007). Furthermore, although not a member of the rhodopsin-like family, the dimer of the seven TMD region of the metabotropic glutamate receptor 1 (Wu et al., 2014) shows cholesterol molecules to be situated such that they make an explicit contribution to the receptor-receptor interface. Moreover, Oates et al. (2012) have shown that cholesterol influences the activity, stability, and oligomerization of the neurotensin NTS1 receptor.

To generalize this to the analysis of the rhombic tetramer model of hM3R is, of course, speculative. Global approaches to sequester cholesterol, for example, by the use of agents such as \( \beta\)-methylcyclodextran, are far too crude to assess this proposed key role of cholesterol in a specific manner. Indeed, when we tried to employ the \( \beta\)-methylcyclodextran–mediated depletion of cholesterol, it was difficult to assess the organization of even the wild-type receptor construct (not shown). Despite these challenges, a number of the other mutants that had substantial effects on receptor organization are also compatible with roles for cholesterol. For example, in the TMD I L80A, I83A, I84A, I87A hM3R receptor mutant, only L80 is predicted to be involved in direct residue-residue interactions in the dimer models we constructed. However, the corresponding residues in these four positions (1.44, 1.47, 1.48, and 1.51) are involved in binding a molecule of cholesterol in both the \( \beta_2\)-adrenoceptor (Cherezov et al., 2007) and serotonin 5-HT2B (Liu et al., 2013).

It is also intriguing to consider the role of helix VIII in receptor organization. As shown, mutation of the leucine residues at positions 8.58 and 8.59 had a large effect on the overall organization of the oligomer. These residues lie next to the cysteine, which, in many receptors, defines the end of helix VIII and is palmitoylated in a regulated fashion (Chini and Parenti, 2009). Regulation of palmitoylation may therefore play an important role in the stability of receptor structure and organization (Chini and Parenti, 2009; Zheng et al., 2012). As with the bulk of post-translational modifications, this is a feature often eliminated from GPCR constructs employed for crystallization to limit heterogeneity of the protein. Although forming the basis for future work, widespread agreement on contributions of helix VIII to interactions among rhodopsin-family GPCRs (Knepp et al., 2012) makes a contribution of lipid acylation a fascinating possibility.

A clear contribution to the organizational structure of residues at the bottom of, but not higher in, TMD IV was also observed, which was consistent with the results of McMillin et al. (2011). This is not an intrinsic feature that is compatible with the rhombic tetramer model shown in Fig. 13. However, it is important to note that Patowary et al. (2013) were unable to discriminate between the rhombic tetramer model and a further higher order complex that might include a hexamer. It is interesting in this regard that some time ago Lopez-Gimenez et al. (2007) developed a daisy chain model of higher oligomers, and the positioning of TMD IV in the rhombic tetramer model is consistent with such a means to extend the oligomer chain.

It is well appreciated that many mutations in GPCRs affect both overall expression levels and the capacity of expressed...
protein to be delivered to the cell surface. As a number of GPCR mutations that are believed to interfere with the effectiveness of homomeric interactions result in the protein being retained within the ER/Golgi apparatus (Salahpour et al., 2004; Lopez-Gimenez et al., 2007; Canals et al., 2009; Kobayashi et al., 2009), it has been suggested on this basis that dimerization of GPCRs is required for cell surface delivery and that effective GPCR dimerization/oligomerization is an integral part of the

Fig. 11. Effects of atropine on the organizational structure and N-glycosylation of hM₃R mutants. (A) The effect of sustained treatment of cells expressing wild-type VSV-SNAP-hM₃R or each of the noted mutants of VSV-SNAP-hM₃R with atropine (10 μM; 24 hours) (filled columns) on the oligomeric organization of the cell surface–delivered receptor is compared with equivalent cells treated with vehicle (open columns). Data represent the slope of the 665-nm hrtFRET signal versus the 620-nm emission signal, with cells expressing wild-type VSV-SNAP-hM₃R treated with vehicle defined as 1.0. ns, not significant; ***P < 0.001; ****P < 0.0001, Student’s t test compared with the untreated samples. (B) Lysates from cells expressing wild-type VSV-SNAP-hM₃R or the noted mutants and treated with atropine or vehicle as in (A) were resolved by SDS-PAGE and immunoblotted with an anti-SNAP antiserum (upper section) or anti-α-tubulin antiserum (lower section).
cellular quality control process. We thus wished to monitor the oligomeric organization of only receptors that had actually reached the cell surface. Traditionally, reporters suitable for resonance energy transfer studies, whether based on bioluminescence resonance energy transfer or FRET, have been added to the intracellular C-terminal tail of the GPCR being studied (Alvarez-Curto et al., 2010a). However, this results in bioluminescence resonance energy transfer studies in particular being limited to reporting on the total population of expressed receptors, whether at the cell surface or located internally. To overcome this, we used an htrFRET-based approach, in which the energy donor and acceptor fluorophores were incorporated covalently into a SNAP tag (Kolberg et al., 2013). This was introduced into the extracellular N-terminal domain of the receptor. Furthermore, we directly measured both cell surface expression by taking advantage of the SNAP tag and protein expression profile via immunoblots to monitor N-glycosylation and protein maturity. These are features routinely linked to effective cell surface delivery (Petaja-Repo and Lackman, 2014). Even for hM3R mutants that displayed poor cell surface expression, this increased in a linear fashion with increasing amounts of cDNA used to transfect cells. We were therefore able to assess receptor-receptor interactions at the cell surface for each mutant compared with the wild-type receptor construct at equal levels of the cell surface receptor. Most importantly, the slope of the line of energy transfer signal corresponding to receptor-receptor interactions against cell surface expression levels provided a measure of the effects on expression levels. Systematic use and analysis of this feature provided us with new insights into the most likely quaternary organization.

Pharmacological chaperones have been widely discussed for their potential to treat disorders in which mutations of GPCRs result in intracellular retention and/or poor protein folding (Nakamura et al., 2010; Maya-Nunez et al., 2012). Ligands with such capacity have also been used to rescue receptors that have defects in oligomer organization to the cell surface. At least in the context of heterologous expression, even wild-type receptors can be chaperoned to the cell surface to increase functional density by the use of ligands able to penetrate the cell and bind to the retained or improperly folded receptor (Leskela et al., 2007). It was therefore interesting to note that sustained treatment of cells expressing various mutants of hM3R with the standard muscarinic receptor antagonist/inverse agonist atropine was able to enhance cell surface levels and the N-glycosylation status of many of the mutants in these studies. Most interestingly, atropine treatment also generally resulted in alteration in the oligomeric organization of these receptor mutants, with the majority now having organization akin to the wild-type receptor. It is further interesting to note in this regard that in FRET-based studies on the organization of the β2-adrenoceptor reconstituted into a model lipid bilayer, Fung et al. (2009) generated results favoring constitutive tetrameric receptor organization. They also observed that the addition of an inverse agonist ligand led to tighter packing of protomers and/or the formation of more complex oligomers by

**Fig. 12.** Molecular models of alternative hM3R oligomeric arrangements. The figure illustrates three distinct, speculative, but conceptually possible oligomeric molecular models of an hM3R tetramer: rhombic (A), linear (B), and square (C).
reducing conformational fluctuations in individual protomers (Fung et al., 2009). Atropine is a muscarinic blocker that reduces constitutive basal activity and is therefore an inverse agonist. It is likely that many receptor mutants that are poorly expressed contribute to disease, and those that have been suggested to be potentially suitable for recovery by treatment with pharmacological chaperones are actually oligomeric organization–deficient rather than lacking a capacity to bind the ligand. As mutations that have such effects are observed in positions throughout the receptor protein sequence, the idea that externally facing residues in many helices can contribute to the quaternary organization of a receptor is entirely consistent with both the model and experimental data.

Overall, these studies provide clear evidence that an incorrect organizational structure of many mutants of hM₃R provides the molecular basis for why they are poorly expressed and fail to be effectively trafficked to the cell surface. They also therefore provide further support for models in which dimerization/oligomerization is an integral early step in cellular recognition and application of protein quality control. Moreover, based on molecular modeling studies that have
allowed testable hypotheses, the mutagenesis studies performed herein suggest that a substantial organizational feature of hM3R, at least when expressed in such a heterologous system, may be a tetramer with a rhombic shape. They also provide insight into the potential contribution of molecules of cholesterol and indeed possibly regulated palmitoylation to the overall organization and potential stability of GPCR oligomers. It will be particularly important to develop ways to explore this specific suggested role of cholesterol without the need to generally deplete levels of this lipid. It will also be fascinating to explore whether this model of rhombic organization of receptor tetramers is broadly applicable across the family of rhodopsin-family GPCRs.

Authorship Contributions

Performed in research design: Varela Liste, Caltabiano, Ward, Alvarez-Curto, Marsango, Milligan.

Conducted experiments: Varela Liste, Marsango, Ward.

Contributed new reagents or analysis tools: Caltabiano, Ward, Alvarez-Curto.

Performed data analysis: Varela Liste, Caltabiano, Ward, Alvarez-Curto, Marsango, Milligan.

Wrote or contributed to the writing of the manuscript: Varela Liste, Caltabiano, Ward, Alvarez-Curto, Marsango, Milligan.

References


Schrodinger LLC, Cambridge, MA.


Biochemistry 45:5588–5604.

Biochemistry 41:5588–5604.

Biochemistry 41:5588–5604.

Address correspondence to: Graeme Milligan, Wolfson Link Building 253, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K. E-mail: Graeme.Milligan@glasgow.ac.uk or Gianluigi Caltabiano, Unitat de Bioestadistica, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. E-mail: gianluigicaltabiano@gmail.com